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Effects of exogenous abscisic acid on the expression of citrus fruit ripening-related genes and fruit ripening

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ABSTRACT

Despite exogenous abscisic acid (ABA) treatment is a widely-used effective method to prove its role in plants, this method has been limitedly explored in citrus fruits. Here, we examined the effects of exogenous ABA and nordihydroguaiaretic acid (NDGA) on the color index H value and the contents of soluble sugars, organic acids and endogenous hormones in the fruits of *Citrus reticulata Blanco cv.* Ponkan. Additionally, the expression profiles of genes involved in the ABA, sugar and organic acid metabolism and signal transduction pathway in fruits were also investigated. The results indicated that exogenous ABA could accelerate fruit coloring, significantly decrease the organic acid content, and affect the ripening of citrus fruit, while treatment with NDGA could restrain fruit coloring and acid degradation. Exogenous ABA treatment produced minor effect on the accumulation of sugars but largely regulated the expression of most sugar- and acid-related genes, indicating a coordinate interaction between the ABA signaling pathway and sucrose signaling pathway to regulate citrus fruit ripening. Especially, *CsSUC 3* plays a vital role in accumulation of sugars during the last ripening stage of citrus fruit, which could be induced by ABA treatment. The transcript levels of *CsACO1* and *CsNADP-IDH* in ABA-treated fruits were significantly higher than in the control fruits during the late ripening stages, which was conducive to the degradation of organic acids. Moreover, ABA could restrict its own accumulation at 14 DAY not only by inducing the expression of *ABA 8′-hydroxylase 1* but also by reducing the expression of *CsNCED1*, and the transcriptional levels of other ABA-related genes were largely triggered by exogenous ABA. Overall, ABA is a positive regulator of ripening and could be used to regulate citrus fruit ripening effectively.

1. Introduction

The fleshy fruits have been divided into two groups with contrasting ripening patterns. Climacteric fruits (such as tomato, banana, peach and apple) show a burst in ethylene release and an increase in respiration rate during ripening, but not the non-climacteric fruits (such as citrus, strawberry and cherry; White, 2002; Leng et al., 2014). Ethylene remains the most explored hormone owing to its predominant role in the ripening of climacteric fruits (Alexander and Grierson, 2002; Kevany et al., 2007; Liu et al., 2015), while abscisic acid (ABA) seems to have a stronger role in the ripening of non-climacteric fruits (Jia et al., 2011; Wang et al., 2013; Nicolas et al., 2014).

So far, numerous studies have been performed about the role of ABA in the regulation of fruit ripening. A rapid increase of ABA content was found during fruit ripening in both non-climacteric (Romero et al., 2012a; Wang et al., 2013) and climacteric fruits, prior to the peak of ethylene (Zhang et al., 2009a). Recently, Zhang et al. (2009b) found that the exogenous ABA could increase endogenous ABA levels both in pulp and seed, inducing the expression of ethylene-related genes and promoting tomato fruit ripening. However, the treatment with NDGA showed the opposite results, delaying fruit ripening. Additionally, the application of ABA accelerated fruit coloring and ripening in strawberry. Silencing a 9-cis-epoxycarotenoid dioxygenase gene (*FaNCED1*) encoding a key ABA synthesis enzyme, using TRV- mediated VIGS, caused a decrease in ABA content and uncolored fruits. More importantly, this uncolored phenotype could be rescued by exogenous ABA (Jia et al., 2011). The ABA-deficient mutant fruit provides an ideal experimental system to explore the role of ABA in the regulation of fruit ripening. Pinalate, a spontaneous fruit-specific ABA-deficient mutant from the 'Navelate' orange (*Citrus sinensis L. Osbeck*), shows

Abbreviations: ABA, abscisic acid; NDGA, nordihydroguaiaretic acid; DAY, days after the first treatment; DAF, days after flowering; qRT-PCR, quantitative real-time polymerase chain reaction.

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a delay on fruit degreening (Rodrigo et al., 2003). Another ABA-deficient mutant, *high-pigment 3* (*hp3*), was obtained in tomato. The lack of ABA leads to an abnormal increase of the plastid compartment size and the accumulation of 30% more carotenoids in the mature fruit (Galpaz et al., 2008). These results show that ABA plays a crucial role in the regulation of fruit ripening.

Recently, the underlying molecular basis of this control in fruits has attracted more and more attention. Two different ABA receptors from strawberry, *FaCHLH/ABAR* and *FaPYR1* (Chai et al., 2011; Jia et al., 2011), and one receptor from grape, *VvPYL1* (Li et al., 2012), were determined to play positive roles in ABA-mediated fruit ripening. Jia et al. (2013b) found that up- and down- regulation of *FaABI1* (the type 2C protein phosphatase) expression levels delayed and advanced fruit ripening, suggesting its negative role in strawberry fruit ripening. More recently, another signal component, *FaSnRK2.6*, functioning downstream of PYR/PYL and PP2Cs, was also found to be a negative regulator during strawberry fruit development and ripening (Han et al., 2015). In addition, a transcription factor, *VvABF2*, has been proven to be involved in several ABA-mediated ripening-related pathways, positively regulating grape fruit ripening process (Nicolas et al., 2014). These results provide some insights into the molecular mechanism of fruit ripening regulatory networks in response to ABA. Despite great advances in the research on the role of ABA in the regulation of fruit ripening, little information is available regarding the ABA-mediated ripening controlling mechanism in citrus.

Currently, the approach to explore the regulation of fruit ripening is very limited in citrus. Specific citrus materials, such as spontaneous late-ripening mutants and color mutants, are usually used in the study of citrus ripening (Rodrigo et al., 2003; Liu et al., 2006a). In the present work, an experiment with a widespread application prospect in the actual production was conducted on Jinshuigan (*Citrus reticulata Blanco cv.* Ponkan). We examined the effects of exogenous ABA and NDGA on the fruit ripening-related quality and endogenous hormones content. The expression profiles of genes involved in the ABA, sugar and organic acid metabolism and signal transduction pathway in fruits treated with ABA and NDGA treatment were also investigated.

2. Materials and methods

2.1. Plant material and ABA treatment

Jinshuigan (*Citrus reticulata Blanco cv.* Ponkan) used in this study was cultivated in the Fruit and Tea Institute of Hubei Agricultural Science Academy, Hubei Province, China. Fruits before color breaker stage (190 DAF) in nine grown trees were divided into three groups, treated with 500 μM ABA, 500 μM nordihydroguaiaretic acid (NDGA), and distilled water as a control, respectively. The treatment was repeated every week to ensure high ABA levels during fruit ripening, till the fruits were fully colored. The optimal treatment concentration and efficacy duration of ABA and NDGA for citrus were selected based on the results of a pretest shown in Fig.S1 in the supplementary material. Samples were harvested at four ripening stages, breaker (BK), colored (C), full colored (FC), and full ripening (FR), which were defined according to the previous report (Romero et al., 2012a), corresponding to 0, 14, 26 and 35 days after the first treatment (DAY), respectively (Fig. 1A) . For each stage, 8 fruits were sampled from each tree, and a total of 72 fruits (9 × 8) were collected from 9 trees. After separating from the peels, the pulps were rapidly frozen in liquid nitrogen and kept at −80 °C for further analysis.

2.2. Peel color measurement

The citrus peel color of each fruit was measured at three evenly distributed equatorial sites using the CIELAB color system of a MINOLTA CR-400 chromameter (Japan). Eight fruits were measured for each group, twenty-four sites (3 × 8) in total. H values represent hue angle, and if $a > 0$ and $b > 0$, $H = \tan^{-1}(b/a)$; if $a < 0$ and $b > 0$, $H = 3.14 + \tan^{-1}(b/a)$ (a, ±yellow/green; b, ±red/blue). H values vary from 0 to 3.14, standing for purple (0), orange red (0.39), red (0.78), orange (1.17), yellow (1.57), yellow–green (2.09), green (2.61) and blue (3.14), respectively.

2.3. Analysis of soluble sugars and organic acids

The soluble sugar and organic acid composition and concentrations were determined by using an Agilent 6890 N gas chromatography as described by Bartolozzi et al. (1997) with minor modifications. Briefly, 3 g of frozen powder was suspended in 12 ml of chilled 80% methanol in a 75 °C water bath for 15 min. After a 45 min ultrasonic extraction and 4000 × *g* centrifugation for 10 min, the supernatant was collected in a 50 ml volumetric flask. This extraction procedure was repeated two times. Next, each volumetric flask was supplemented with 1 ml internal standard (0.025 g ml^{-1} phenyl-β-D-glucopyranoside, 0.025 g ml^{-1} methyl-α-D-glucopyranoside). Finally, the solution was diluted with 80% ethanol to a final volume of 50 ml and a 0.5 ml of this solution was vacuum concentrated and derivatized for GC analysis. Three replicates were conducted for each sample.

2.4. Determination of endogenous hormone (ABA, JA and IAA) Content

For endogenous hormone extraction, D_6-ABA, H_2JA and D_5-IAA (Icon Isotopes) were used as internal standards for ABA, JA and IAA, respectively, which aimed to calibrate samples. The other procedures were completely followed as described in the report of ABA quantification (Wu et al., 2014). Additionally, the reaction monitoring conditions (Q1/Q3) of JA and IAA and their icon isotopes were described by Pan et al. (2010). High-performance liquid chromatography (HPLC) was used for qualitative and quantitative analysis. Four replicates were performed for each sample.

2.5. RNA extraction and qRT-PCR expression analyses

The pulps sampled from three different trees were mixed and 3 g of the material was used for total RNA extraction according to the procedures described previously (Liu et al., 2006b). After the total RNA with high quality and integrity was extracted, the first-strand cDNA was synthesized using a PrimeScript™ RT Reagent kit with gDNA Eraser (TaKaRa) according to the manufacturer's instructions. Several genes involved in the fruit ripening process, such as genes in ABA metabolism and signal transduction pathways and soluble sugars and organic acids metabolism pathways, were selected for quantitative real-time PCR (qRT-PCR). A part of the gene-specific primers were referred to our previous reports (Wu et al., 2014; Zhang et al., 2014). Sequences of other primer pairs were listed in Supplementary Table S1. The differential expression of the selected genes was validated by using a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems), with the reference of *actin* gene according to Liu et al. (2007). Each well contained 10 μl of the reaction mixture consisting of primers diluted in SYBR Green PCR Master Mix (Applied Biosystems) and cDNA in double-distilled water (ddH_2O). Reactions were set with an initial incubation at 50 °C for 2 min and 95 °C for 5 min, and then 40 cycles at 95 °C for 15 s, 58 °C for 15 s and 72 °C for 30 s. Four technical replications